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| 14. ABSTRACT Tissue inflammation and inflammatory cytokines can positively affect breast cancer prognosis. By providing a detailed understanding of the mechanism of inflammasome formation and activation, we hope to create the potential for novel inflammation based cancer therapies. The protein, NALP, forms the core of the inflammasome complex. The chief domains of NALP do not express well in bacterial or insect cell expression systems, exhibiting poor expression levels and solubility. The NALP pyrin domain has been successfully produced and purified, and initial crystals of this protein have been produced. Constructs of the adaptor CARDINAL were found to express well, but suffer proteolysis during purification. Full-length constructs of NALP have been produced by baculovirus/SF9 expression; have been observed to exist in monomer/oligomer equilibrium in solution. | | | | | |
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Table of Contents

| | <u>Page</u> |
|-----------------------------------|-------------|
| Introduction..... | 4 |
| Body..... | 4 - 8 |
| Key Research Accomplishments..... | 9 |
| Reportable Outcomes..... | 9 |
| Conclusion..... | 10 |
| References..... | 10 |
| Appendices..... | 11 |

Introduction

Breast cancer is a leading cause of cancer-related death worldwide, having a particularly high incidence in the United States. The occurrence of breast cancer is correlated with hereditary and environmental factors, and its prognosis is linked to a large number of proteins and cellular pathways. It has been shown that tissue inflammation and inflammatory cytokines may have an impact on disease development and prognosis^{1,2}. A protein complex termed the inflammasome is capable of triggering tissue inflammation, via the inflammatory cytokine interleukin-1beta (IL-1 β)^{3,4,5}.

The inflammasome complex comprises three separate proteins⁴. A multi-domain protein called NALP (NACHT-domain, leucine-rich repeat domain, pyrin domain-containing protein) forms the core of this complex. The NACHT domain (found in proteins NIAP, CIITA, HET-E and TP1) of NALP is thought to be an ATPase capable of forming ATP-dependent oligomers; the NALP leucine-rich repeat (LRR) domain is thought to bind an activating ligand; and the pyrin domain is death-domain superfamily member thought to form heterodimeric interactions with other death-domains. An adaptor protein, CARDINAL (CARD inhibitor of NF-kB-activating ligands), binds to ligand-activated NALP via the NACHT domain. A second adaptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), is thought to bind to the NALP pyrin domain through its own pyrin domain. The goal of this work is to develop an understanding of the activation of the inflammasome complex at the molecular level, though biophysical and biochemical characterization of the proteins involved.

However, we note that in 2007 a competing group published a body of work that substantially overlaps with the research plan put forth in our original Statement of Work (SOW)⁶. Specifically, the competing work is very similar to SOW Tasks 2, 4, 5, and 6 (biochemical characterization NALP proteins and the inflammasome complex). Consequently, a new SOW covering a new research topic has been prepared. This proposed work involves characterization of the interaction between inhibitory antibodies and the epidermal growth factor receptor (EGFR), a topic with strong relevance to breast cancer. The revised SOW has been approved by the University of Pennsylvania School of Medicine business office and, at the time of writing, is pending approval by the funding agency. Since the revised SOW covers part of the period of this progress report (months 12-24), and since the author does not know when the revised SOW may be finally approved, this report will detail progress towards the tasks described in both the original and the revised SOW. A copy of the proposed revised SOW is included in the Appendix.

Body

The following section details the progress of tasks described in the original Statement of Work.

Task 1. Produce purified proteins. (months 1-24)

The production of pure, intact protein is a prerequisite to further biochemical and biophysical analysis. Consequently, much effort has been given to identifying protein constructs and expression methods that allow generation of significant quantities of stable, pure protein.

Protein expression was initially attempted in *Escherichia coli*. Standard cloning techniques were used to insert genes encoding proteins or domains of interest into plasmids with poly-histidine or glutathione-S-transferase (GST) tags. Sequences of all constructs were verified by PCR (polymerase chain reaction). pET-22b (Novagen) was used for C-terminal his-tagged constructs, a modified version of pET-21a (Novagen) was used for N-terminal his-tagged constructs, and pGEX-4T (GE Healthcare) was used to provide a N-term GST tag. Constructs were expressed in Rosetta2(DE3)pLysS (Stratagene), which carries mammalian codon supplementation. Protein overexpression was induced by 0.5 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) in standard LB (Luria-Bertani) medium, at a culture density of OD₆₀₀ ~0.6. Expression time was dependent on temperature: 3h at 37C, 5h at 30C, or overnight at 20C.

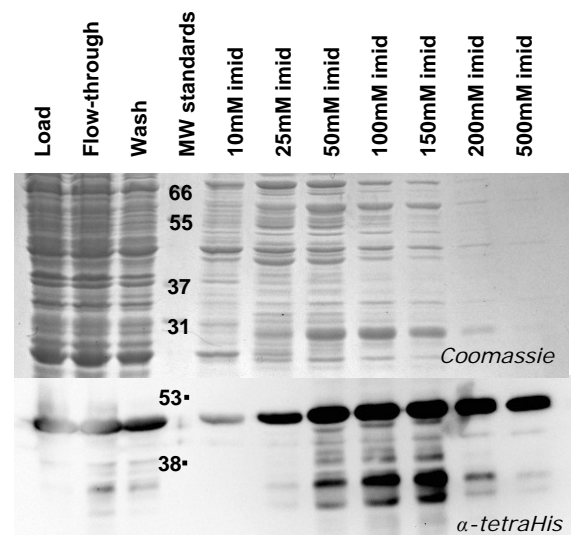


Figure 1. LRR domain from NALP2 expresses at low levels in *E. coli*. Coomassie stained gel (upper) and immunoblot (lower) of imidazole elution fractions from Ni-NTA chromatography of a his-tagged LRR construct. Overexpressed protein is detected by immunoblot, but is not the most abundant species in imidazole fractions. Further, a substantial quantity of the protein remains unbound in the “flow-through” fraction, indicating possible aggregation and poor solubility of the construct. MW standards (kDa) are marked.

Most NALP constructs were found to express poorly in *E. coli*. His-tagged full-length NALP2 and NALP12 are produced at very low levels in *coli*, which suggests that these constructs cannot fold properly in this expression system. His-tagged constructs consisting of the NACHT domain alone behave similarly. His-tagged constructs comprising the LRR domain of NALP2 or NALP12 express in sufficient quantity to be detectable by western blot (Fig. 1), but exhibit poor solubility that may be related to oxidation of a relatively large number of surface exposed cysteine residues. The pyrin domain alone was found to express well in *coli*. C-terminally his-tagged pyrin domains from NALP2 and NALP3 were purified to homogeneity by Ni-NTA (nickel-nitrilotriacetic acid, Qiagen) affinity chromatography followed by size exclusion chromatography (Superose 12, GE Healthcare) (Fig. 2). Purified pyrin constructs concentrated to ~3 - 5 mg/ml are soluble and stable for several weeks at 4°C.

The adaptor protein CARDINAL exists in several isoforms in humans, and a homolog exists fused to the C-term the NALP1 protein. CARDINAL is interesting both because of its function as an adaptor protein in the inflammasome complex, and because the bulk of the protein is not homologous to any previously characterized protein domains, as determined by sequence-based analysis. His-tagged CARDINAL constructs were found to express in *E. coli* in relatively high amounts, and were purified by Ni-NTA affinity chromatography, cation exchange chromatography, and size exclusion chromatography (Fig. 3). However, these CARDINAL were highly susceptible to proteolysis throughout the purification procedure.

Protein expression was also attempted by baculovirus infection of Sf9 (*Spodoptera frugiperda*) insect cells. Expression in Sf9 insect cells utilized the Bac-to-Bac system (Invitrogen) and followed established protocols for intracellular protein⁷. Briefly, constructs were cloned into pFB-HT plasmids, incorporating either a C- or N-terminal his-tag. The pFB plasmids were transformed into DH10bac cells, and, following successful transposition of the gene of interest, viral bacmid was isolated. Bacmid was then transfected into Sf9 cells (Invitrogen), from which virus was harvested and amplified. For protein expression, insect cells were cultured in suspension at 27°C using Erlenmeyer culture flasks or Cytostir bioreactors (Kontes), with Sf-900 II serum free medium (Invitrogen). Culture density, amount of virus, and expression times were varied.

His-tagged constructs consisting of individual NALP domains did not express well in insect cells. LRR constructs exhibit substantial proteolysis, and constructs comprised of the NACT domain express at low levels and rapidly precipitate during subsequent purification steps. However, modest amounts of his-tagged full-length NALP2 was produced and purified from Sf9 culture, by Ni-NTA affinity chromatography followed by size exclusion chromatography (Superose6, GE Healthcare) (Fig. 4).

Task 2. Assay interaction between LRR domain and ligand (months 1-12)

Purified NALP LRR domain is a necessary reagent for LRR/ligand binding studies. However, work done by a competing group of a reconstituted inflammasome indirectly suggests an interaction between LRR and muramyl dipeptide ligand⁶.

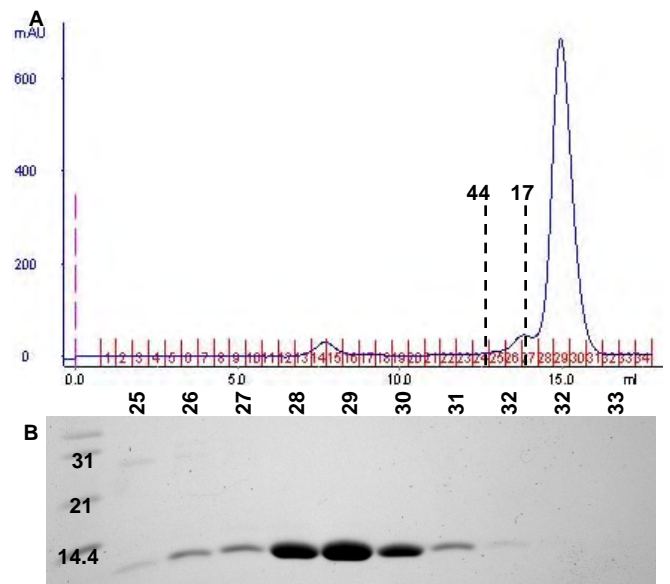


Figure 2. NALP2 pyrin domain is stable as an independent construct, and can be purified to homogeneity. (A) FPLC chromatogram of C-term his-tagged NALP2 pyrin domain run over Superose 12 size exclusion column. Elution positions of sizing standards are noted. (B) Coomassie stained gel of size exclusion fractions, encompassing the protein peak. Molecular weight standards (kDa) as marked.

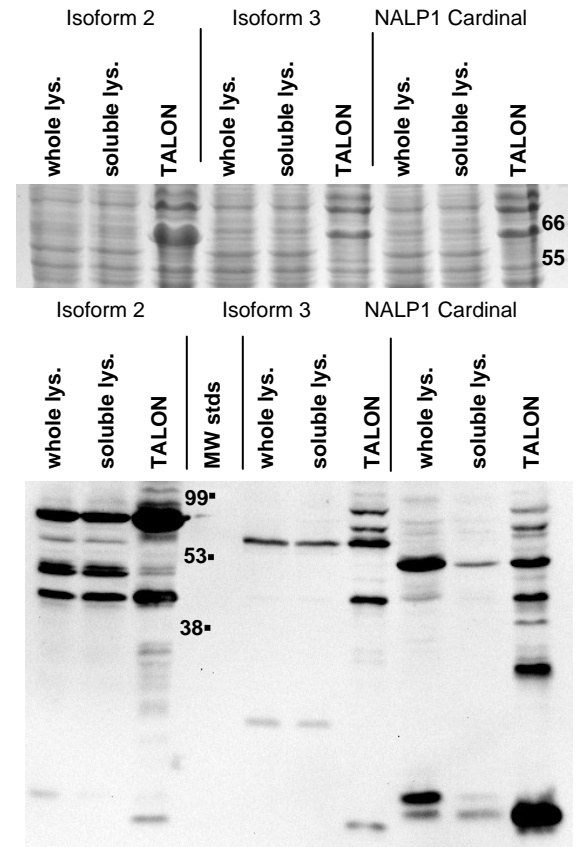


Figure 3. CARDINAL constructs express solubly but suffer proteolysis. Coomassie stained gel (upper) and anti-tetraHis immunoblot (lower) of test expressions of three CARDINAL proteins in Sf9 cells. Whole lysate, soluble lysate, and protein bound to TALON beads, after one PBS wash, are shown. All three constructs express some amount of soluble protein. CARDINAL isoform 3 and the NALP1 CARDINAL domain are heavily degraded. CARDINAL isoform 2 yields a significant amount of protein and is less degraded initially. Molecular weight standards as marked (kDa).

Task 3. Determine X-ray structure of NALP domains and complexes (months 6-36)

Full-length NALP2, purified from Sf9 cells and concentrated to ~1 mg/ml in Superose 6 running buffer (20 mM Tris, 150 mM NaCl, 2 mM BME, 1 mM MgCl₂, pH 7.5). Concentrated protein was subjected to crystallization trials by microbatch under-oil crystallography. Equal volumes of protein and crystallization condition were mixed, using sparse matrix screens from Hampton Research and a custom grid screen developed in-house. Even at this relatively low concentration, full-length-NALP2 was observed to precipitate in the majority of conditions. No protein crystals were observed.

NALP2 pyrin domain alone was purified from *coli* and concentrated to ~2 mg/ml in Superose 12 running buffer (20 mM Tris, 150 mM NaCl, 2 mM BME, pH 7.5). Purified pyrin was used for crystallization trials by microbatch crystallography, using sparse matrix screens. Small crystals (5x5x5 μ m) were observed in a condition consisting of 50% 2-methyl-2,4-pentanediol, 0.2 M ammonium acetate, 50 mM acetate pH 5.0 (Fig. 5). These crystals have not reproduced upon transition to a hanging drop format, for crystal optimization. Crystals were also observed amid protein precipitate, in a condition of 25% PEG3350, 100 mM calcium chloride, 50 mM acetate, pH 5.0. These very thin plate-like crystals (~1x10x100 μ m) form reproducibly, as does the heavy precipitate in this condition, but the crystals have been not been optimized to sufficient quality for data collection.

Task 4. Assay ATPase activity of NACHT domain (months 6-24)

Small amounts of full-length NALP (which includes the entire NACHT domain) have been produced. However, ATPase activity has not yet been measured. A simple colorimetric ATPase assay was attempted, using 1mM ATP, 1mM MgCl₂, 1uM NALP (CytoPhos Phosphate Assay BIOCHEM KIT, Cytoskeleton), but no activity was detected. The limited stability of the protein at temperatures above 4C and the limited sensitivity of the colorimetric assay may limit this approach to ATPase activity detection.

Task 5. Characterization NALP oligomerization state (months 6-24)

An initial characterization of purified full-length NALP2 was performed, in the presence of 1 mM MgCl₂, with no added ATP or ADP, by size-exclusion chromatography at pH 7.5 (Superose 6, GE Healthcare) (Fig. 4). Under these conditions, the majority of the protein ran at a size consistent with monomer, with a broad shoulder at a lower elution volume, suggesting equilibrium between monomer and oligomer. Lower molecular weight proteolysis products were also detected.

Task 6. Assay inter-domain and protein-protein interactions (months 6-36)

Intact and stable reagents, particularly CARDIAL and ASC proteins, have not yet been produced, which will be required for these assays.

The following text details progress of tasks described in the proposed revised statement of work.

Task 1. Produce and purify proteins. (months 18-36)

Several monoclonal antibodies have been acquired as pure protein

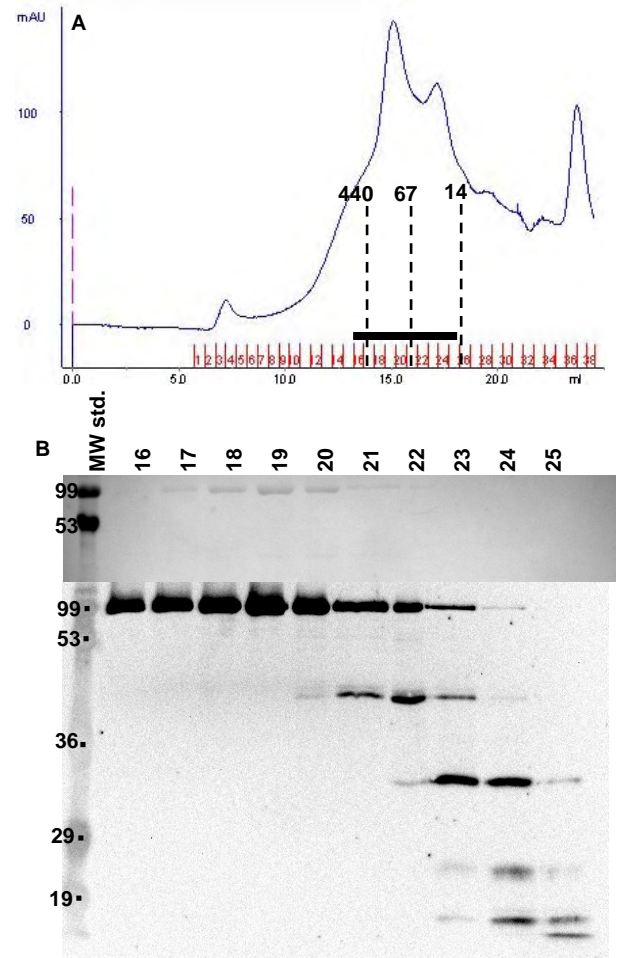


Figure 4. Full-length NALP2 can be purified to homogeneity from Sf9 cells, and exists in a monomer-oligomer equilibrium. (A) Chromatogram of NALP2, expressed in Sf9 cells, elution profile from a Superose 6 size exclusion column. Black line denotes fractions that were further analyzed by SDS-PAGE. Elution volumes of size standards are noted (kDa). (B) Ponceau (upper) and anti-tetraHis immunoblot (lower) showing elution fractions from chromatogram in A. NALP2 is present at low concentration, but is separated from its degradation products. A peak and a shorter elution volume shoulder correspond to elution of full-length NALP2, suggesting monomer-oligomer equilibrium.

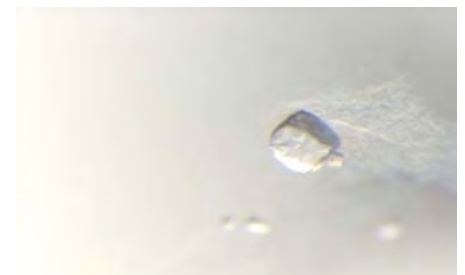


Figure 5. Purified NALP2 pyrin domain forms crystals in 50% 2-methyl-2,4-pentanediol, 0.2 M ammonium acetate, 50 mM acetate pH 5.0.

through existing collaborations with the Ferguson lab. These include mAb C225 (ImClone), mAb 425 (Genentech), mAb 108 (J. Schlessinger, Yale), and 13A9 (Genentech). Fab fragments have been generated from these antibodies by papain cleavage and initially isolated by protein-A column affinity (Fab Preparation Kit, Pierce). Fab fragments are further purified by size exclusion chromatography (SEC250, BioRad).

Additionally, several llama VHH antibody fragments, designated IA1 and 9G8 (R. Roovers, Utrecht University), have been purified. DNA encoding these proteins was cloned into expression plasmid pET-22b (Novagen), resulting in periplasmically targeted C-terminally his-tagged constructs. Protein were overexpressed in Rosetta2(DE3) *E. coli*, by 4h IPTG induction. Cells were pelleted and resuspended in PBS, and VHH antibody constructs were liberated from the periplasmic space by freeze-thaw. Constructs were purified from periplasmic isolate by Ni-NTA (Qiagen) affinity chromatography and size exclusion chromatography (Superose12, GE Healthcare).

The extracellular region of the epidermal growth factor receptor (sEGFR) was produced as a secreted protein by baculovirus infection of Sf9 cells, as described^{8,9}.

Task 2. Assay interaction between antibody inhibitors and sEGFR (months 20-30)

Theh interaction between Fabs and sEGFR was assayed by surface plasmon resonance studies (SPR, Biacore). Dissociation constants were calculated by measuring the equilibrium binding response of free sEGFR to immobilized Fab, over a range of concentrations of sEGFR. Data were fit by nonlinear regression to a Langmuir binding isotherm (Table 1).

| Fab | C225 | 425 | 108 | 13A9 | EGF |
|-----|--------------|---------------|---------------|--------------|------------|
| Kd | 2.3 ± 0.3 nM | 46.4 ± 8.7 nM | 19.8 ± 2.2 nM | 1.1 ± 0.1 nM | 130 ± 4 nM |

Table 1. Measured dissociation constants for the interaction between inhibitory antibodies (or ligand) and sEGFR. Fab or ligand was immobilized on an SPR chip by amine coupling. Varying concentrations of free sEGFR was flowed over the chip, and the equilibrium binding response was measured at each concentration.

Task 3. Assay ability of inhibitor to compete with ligand/sEGFR binding (months 24-36)

Initial characterization of Fab competition with ligand binding has been accomplished using SPR techniques. A saturating concentration of sEGFR, 600 nM, along with a 10-fold molar excess of Fab or EGF ligand, was passed over immobilized EGF ligand. The resulting binding response as measured and compared to the response in the absence of Fab or free ligand (Fig. 6). All Fabs were observed to inhibit the ligand/sEGFR interaction to some degree.

Task 4. Characterize binding footprints of inhibitory antibodies (months 24-30)

An existing panel of sEGFR point mutations, located on domain III, has been assembled, based on prior work characterizing two inhibitory Fabs, C225⁸ and EMD72000⁹, which is a humanized version of 425. The dissociation constants for the interaction of these mutants with the inhibitory antibodies under study will be measured by SPR.

Task 5. Structurally characterize llama VHH inhibitors by X-ray crystallography (months 20-36)

The VHH antibody IA1 was found to express in quantities suitable for crystallographic studies, and was subjected to crystallization trials. Protein at 15 mg/ml was found to form rod-shaped crystals by hanging-drop vapor diffusion, when mixed 1:1 with a reservoir solution of 30% PEG8000, 0.2 M ammonium sulfate, 0.1 M MES, pH 6.0. Crystals were harvested directly from the drop, and flash frozen in liquid nitrogen. IA1 crystals diffracted to 1.55Å, and the structure was determined by molecular replacement, using an existing structure of a llama VHH fragment (Protein Data Bank ID 1i3v) as a search model (Fig. 7).

The structure of IA1 alone gives little insight into its inhibitory activity against the extracellular domain of EGFR. However, the structure of this antibody fragment

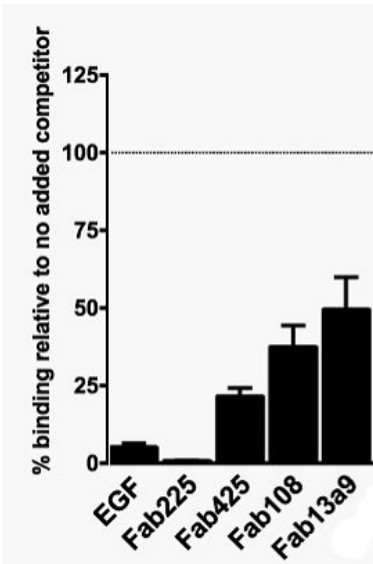


Figure 6. Inhibitory Fabs compete with EGF ligand for binding to sEGFR. 600 nM of sEGFR, plus a 10-fold excess of the indicated Fab, or EGF, was passed over a surface of immobilized EGF. Residual binding is shown as a % of equilibrium binding in the absence of free Fab or EGF.

may have utility as a molecular replacement search model for a future dataset of IA1 in complex with the receptor. Additionally, the unbound form of the antibody will serve as an interesting comparison to the bound form, should the complex structure be determined.

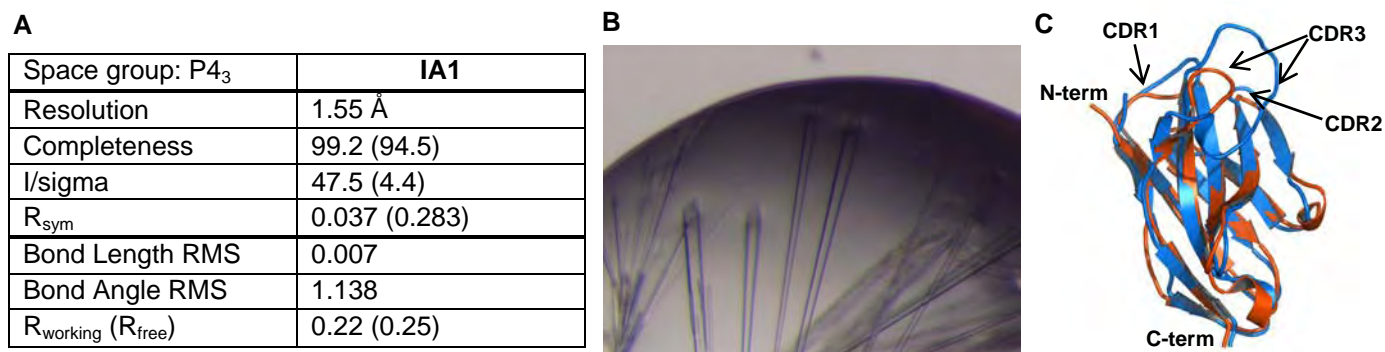


Figure 7. Crystal structure of IA1 llama V_{HH} antibody. The structure of antibody IA1 was determined to a resolution of 1.55 Å. Data were collected at the Advanced Photon Source, beamline 23 ID-B. Data collection and refinement statistics (for the current stage of refinement) are given in A. Values in parenthesis reflect the highest resolution shell of data. RMS = root mean square deviation. (B) Rod-like crystals (75x75x500 μm) formed in 30% PEG8000, 0.2M ammonium sulfate, 0.1 M MES, pH 6.0. (C) Cartoon representation of IA1 (blue) overlaid with the N-terminal Ig domain of the C225 heavy chain (from PDB 1yy8). Differences between the two structures reside chiefly in the complementarity determining region (CDR) loops. Particularly, CDR3 is much longer in IA1 and folds against the surface of the Ig domain.

Key Research Accomplishments

Inflammasome Characterization (original SOW)

- Multi-microgram amounts of full-length NALP2 have been produced in Sf9 cells and purified to homogeneity.
- The NALP pyrin domain has been purified from *E. coli*, and initial crystals of this domain have been grown.
- Full-length NALP has been assessed to be in apparently monomer/oligomer equilibrium by size exclusion chromatography.

Epidermal growth factor receptor interaction with inhibitory antibodies (revised SOW, months 18-24)

- A panel of inhibitory Fab fragments has been purified and can be used for subsequent biochemical experiments.
- Affinities for these binding of these Fab fragments to sEGFR have been quantitated.
- Inhibitory Fab fragments have been shown to compete with EGF ligand for binding to sEGFR.
- The crystal structure of one llama V_{HH} domain, IA1, has been determined in the unbound state.

Training Accomplishments

- The PI has acquired proficiency in standard cloning techniques, protein expression methods in *E. coli* and Sf9 cells, and protein purification techniques.
- The PI has attended the Analytical Ultracentrifugation Workshop at the National Institutes of Health, funded by The Foundation for Advanced Education in the Sciences. This workshop focuses on experimental design and data interpretation for hydrodynamic and thermodynamic techniques, including sedimentation equilibrium and sedimentation velocity analytical ultracentrifugation, dynamic light scattering, and isothermal titration calorimetry. These techniques all have strong relevance to the PI's field of study and to the aims of the project funded by this grant.
- The PI has been accepted by the National School on Neutron and X-ray Scattering, held at Argonne National Laboratory and Oak Ridge National Laboratory. This is an intensive fifteen day course on scattering techniques, taught by experts in the field. Neutron and X-ray scattering methods are powerful biophysical tools for the characterization of biological molecules, and serve as a useful complement to high resolution structural characterization of proteins and protein interactions.
- The PI has acquired extensive experience in X-ray crystallography techniques, including instrument operation, data collection, and data processing with in-house X-ray generator/diffractometers, as well as high flux X-ray beamlines at synchrotrons radiation facilities. This experience includes extensive hands-on training on over 10 data collection trips with the Ferguson laboratory to beamlines at MacCHESS (Macromolecular Diffraction Facility at the Cornell High Energy Synchrotron Source), NSLS (National Synchrotron Light Source), and APS (Advanced Photon Source).
- Additionally, the PI has gained experience with SAXS (small angle X-ray scattering) from several trips with the Ferguson lab to MacCHESS.
- The PI has achieved proficiency with the algorithms and software used for crystallography and SAXS data collection and processing. This includes determination of X-ray crystal substructure by molecular replacement and experimental phasing methods, refinement of crystallographic models, and molecular envelope calculation from X-ray scattering data.

Reportable Outcomes

Problems with the expression and purification of protein reagents required to characterize the NALP inflammasome have limited the amount of potentially publishable data collected this far.

With respect to the data collected on antibody inhibitors of the extracellular domain of EGFR, a manuscript is in early stages of preparation that will detail binding affinities of these antibodies, comparison of their ability to inhibit binding of EGFR to ligand, comparison of their ability to inhibit binding of other inhibitory antibodies, as well as epitope of these antibodies mapping using a series of sEGFR point mutants.

Conclusions

Understanding the molecular mechanism of inflammasome formation and activation will yield valuable insight into tissue inflammation, a phenomenon linked to breast cancer prognosis. Production of sufficient quantities of intact, soluble protein continues to be a major focus and challenge in this work. A NALP pyrin domain has been purified, and may be a useful reagent in investigating the interaction between NALP and the adaptor protein ASC. The ASC pyrin domain may express well in *coli*, and may be used to test the interaction between the pyrin domains of these two proteins. The LRR domain of NALP is a critical component for the biochemical characterization of the interaction between NALP and potential ligands. Additional expression conditions may improve the yield and behavior of LRR domain constructs. Expression in low oxygen/anaerobic conditions may increase soluble yield by reducing the oxidation of cysteine residues, as may very high concentrations of reducing agent. Because the boundaries of the LRR and NACHT domains are not readily evident from sequence analysis, it may be advantageous to try expressing additional constructs with different N- and C-termi. Additionally, use of a solubility enhancing tag, such as maltose binding protein, may ameliorate the poor expression and solubility of the NACHT and LRR domains. Finally, lactose-based overnight autoinduction methods have been shown to improve yield and quality for some *coli* expressed protein¹⁰, and may give better results than standard IPTG-induced overexpression in LB.

Work towards the characterization of antibody-based EGFR inhibitors offers a different avenue towards combating breast cancer. We have collected an initial set of data that helps to characterize a set of inhibitory antibodies. Purified Fab and V_{HH} fragments, and sEGFR constructs, will be used to further probe these interactions both in solution and through structural biology.

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Appendix

Statement of Work

Task 1. Produce and purify proteins. (months 18-36)

- Acquire partially purified monoclonal IgG antibodies through existing collaborations (months 18-24)
- Cleave IgG and isolate antibody F_{ab} fragments (months 18-24)
- Acquire genes for llama V_{HH} antibody fragments through existing collaboration (months 18-22)
- Clone llama V_{HH} fragments, optimize bacterial expression and purification strategy (months 20-24)
- Produce and purify panel of llama V_{HH} antibody fragments (months 20-36)
- Produce and purify sEGFR, single-domain sEGFR constructs, and variants (months 20-36)

Task 2. Assay interaction between antibody inhibitors and sEGFR (months 20-30)

- Surface plasmon resonance studies between F_{ab} and sEGFR (months 20-25)
- Surface plasmon resonance studies between V_{HH} and sEGFR (months 25-30)
- Sedimentation equilibrium analytical ultracentrifugation studies (months 25-30)

Task 3. Assay ability of inhibitor to compete with ligand/sEGFR binding (months 24-36)

- Competition between antibody inhibitors and ligand (months 24-30)
- Competition between individual antibody inhibitors (months 30-36)

Task 4. Characterize binding footprints of inhibitory antibodies (months 20-30)

- Screen inhibitors against panel of sEGFR surface residue mutants (months 20-30)
- Design and generate new sEGFR point mutations based on crystallographic data (months 24-30)
- Screen inhibitors against second panel of sEGFR surface mutants (months 30-36)

Task 5. Structurally characterize llama V_{HH} inhibitors by X-ray crystallography (months 20-36)

- Identify crystallization conditions for V_{HH} inhibitors (months 20-24)
- Identify crystallization conditions for inhibitor/sEGFR complexes (months 24-30)
- Test crystals for diffraction (months 20-30)
- Collect high-resolution diffraction data (months 24-36)
- Build and refine models of inhibitors and complexes (months 24-36)
- Analyze crystal structures (months 24-36)
- Characterize conformation of inhibitor/sEGFR complexes by DLS and SAXS (months 24-30)